

# Use of Blue Cotton for Detection of Mutagenicity in Human Feces Excreted after Ingestion of Cooked Meat

by Hikoya Hayatsu,\* Toshiko Hayatsu,\* and Yusuke Wataya\*

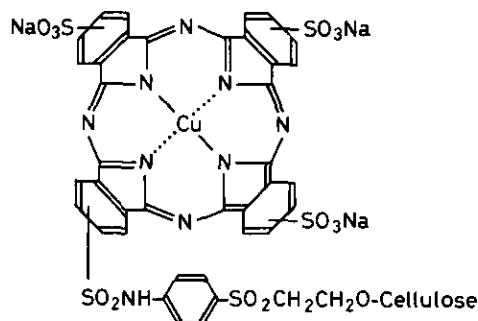
Fried ground beef has been shown to contain mutagens, and the major mutagenic component has been identified as 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). Mutagens in feces of three adult volunteers were fractionated by treatment of the feces with blue cotton followed by chromatography on a carboxymethyl cellulose column. The chromatographic fraction corresponding to MeIQx in terms of the position of elution was examined for mutagenicity in *S. typhimurium* TA 98 with metabolic activation. When meals containing no heated meat were eaten, this fraction of feces showed little or no mutagenicity. On eating fried ground beef, the feces excreted in the next 2 days showed greatly increased mutagenicity in this fraction. By eating no-meat meals subsequent to the meat meal, the mutagenicity resumed the original low level on the fourth day after the meat meal. The components in the mutagenic fraction were analyzed by high-pressure liquid chromatography, and were shown to differ from MeIQx.

## Blue Cotton

Blue cotton is absorbent cotton bearing covalently linked trisulfo-copper-phthalocyanine residues (1). It can adsorb in a specific manner organic compounds having three or greater numbers of fused aromatic rings (1). It is believed that the adsorption takes place by means of hydrophobic interactions between the copper-phthalocyanine nucleus and the aromatic substances. There is evidence for this interaction: the visible spectrum of tetrasulfo-copper-phthalocyanine undergoes peak shifting on mixing with these compounds (unpublished work). Since many carcinogens and mutagens belong to this class of compounds, blue cotton is useful to isolate these compounds from crude materials. Mutagens which can be produced by cooking foods belong to this class (2) and they have strong affinity to blue cotton (1). As a result, blue cotton is especially suited for the isolation of mutagens from cooked foods such as fried ground beef (1,3). We show here that blue cotton is also useful for isolating mutagens from human feces excreted after ingestion of cooked meat.

## Fecal Mutagenicity and Mutagens in Cooked Meat

Mutagens in human feces have been a subject of intensive study (4). One of the purposes of these studies



Blue Cotton

is to seek causes for colon cancer. Recently, fecapentaenes, which show mutagenicity towards *Salmonella typhimurium* TA 100 without metabolic activation, were isolated from human feces (5,6). The fecapentaenes are products of intestinal *Bacteriodes*, and investigation of the human populations gave no relationship between the fecal occurrence of these compounds and colon cancer (7). Furthermore, although the etiology of colon cancer is attributed to diet (8), there has been no report which relates specific diets to fecal mutagens except for a statistical study showing that non-vegetarians have higher fecal mutagenicity than vegetarians (9).

On heating beef at high temperatures during cooking,

\*Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan.

mutagens are formed. Two compounds that are strongly mutagenic towards bacteria have been detected in cooked beef (2,3,10-14): 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) (10) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (15). It can therefore be expected that human fecal mutagenicity would increase on eating fried ground beef.

## Extraction of Mutagens from Cooked Beef

Ground beef (1 kg) was mixed with diced onion (330 g), one egg (50 g), bread crumbs (30 g), salt (5 g), and flavored with ground pepper and nutmeg. Twenty patties, each approximately 1 cm in thickness, were produced from this mixture and were fried in an iron pan over a gas flame. The temperature of heating was monitored by a thermocouple. During the frying, the patty was turned over at every minute and cooked well-done over a period of 8 min. The temperature inside the patty near the interface between meat and pan surface was 80 to 90°C for the first 5 min, and 90 to 110°C for the next 3 min. A raw patty of 65 g weighed 39 g after the cooking.

Mutagens were extracted from the cooked beef (100 g raw meat equivalent) by homogenization with 300 mL of water. The homogenate was centrifuged at 9000 rpm for 30 min. The supernatant was treated with blue cotton (obtained from Funakoshi Pharmaceutical Co., 2-3 Surugadai, Kanda, Tokyo) as described below for feces slurry, and a portion (20 g raw meat equivalent) of the extract obtained was subjected to carboxymethyl (CM)-cellulose column chromatography. The column preparation was described previously (3). The volume of each fraction was 3 mL. Fractions 1-3 were eluted with dilute formic acid at pH 3.0, fraction 4 with water, fractions 5-7 with 50% methanol, and fractions 8-10 with 50% methanol-ammonia at pH 11.0. The mutagenicity of each fraction was assayed on *S. typhimurium* TA 98 with S-9 mix, as described (3). The background level of reversion,  $28 \pm 5$ , had been subtracted from the observed numbers of revertants.

## Extraction of Mutagens from Feces

Mutagens were extracted from fresh feces (100-200 g) by mixing with water (350 mL) and homogenizing for 5 min in a blender. The homogenate was centrifuged at 9000 rpm and 20°C for 30 min. The slurry collected by decantation was treated twice with blue cotton (1 g and 0.5 g of the cotton used, with 30-min shaking for each treatment). The combined cotton was washed with water, extracted with methanol-ammonia (50:1) (130 mL  $\times$  2), and the pooled extracts were flush-evaporated at 30°C. The residue was dissolved in dimethyl sulfoxide (0.6 mL) and the solution was mixed with water (50 mL). The aqueous solution was then treated with blue cotton (0.1 g  $\times$  2) and the cotton was extracted with methanol-ammonia (30 mL  $\times$  2). The material obtained

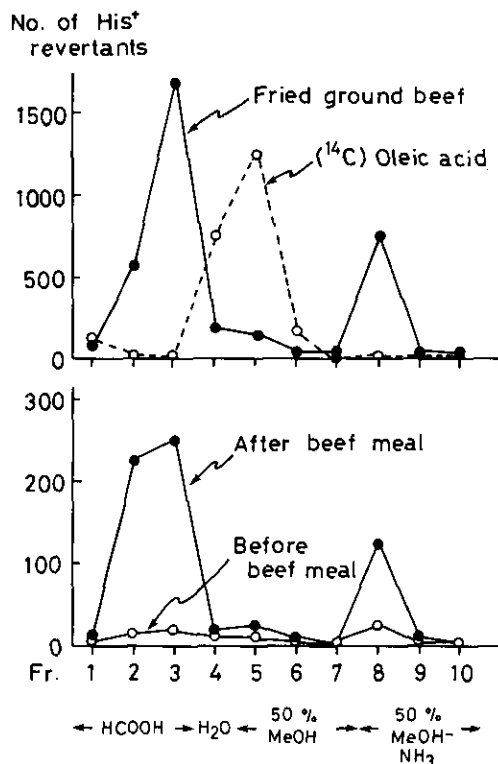


FIGURE 1. Carboxymethyl cellulose column chromatography of mutagenic components originating from cooked ground beef. (a) Mutagens extracted from cooked beef. A standard [<sup>14</sup>C]oleic acid was separately chromatographed, which was monitored by radioactivity. (b) Mutagens extracted from human feces before and after ingestion of fried ground beef. The fecal samples used in the experiments shown here were those collected on day 4 (○) and on day 5 (●) of the time-course study illustrated in Fig. 2 (curve A<sub>1</sub>). Standards of MeIQx eluted in Fractions 2-3 and IQ in Fraction 8.

on evaporation of the solvent was subjected to the CM-cellulose chromatography.

## Mutagenicity Arising on Ingestion of Cooked Beef

A healthy Japanese female (designated A), age 45, ate meals containing no heated meat or heated fish for 3 ½ days. During the evening of the day 4, she ate a meal containing 150 g (raw weight) of fried ground beef (~10% fat). Meals on days 5 to 8 again lacked cooked meat or fish. The entire feces from days 3 to 8 were individually analyzed. Figure 1 shows profiles in the CM-cellulose column chromatography of an extract of fried ground beef (a) and of extracts of feces before and after eating the fried ground beef (b). It is seen that the Fractions 2 and 3 of the fecal sample contained mutagenic material. These fractions corresponded to the major mutagenic fraction of extracts obtained from cooked ground beef. The mutagens in fecal samples obtained after the ingestion of ground beef greatly increased in Fractions 2 and 3. Standard samples of MeIQx and IQ were eluted in Fractions 2-3 and Fraction 8, respec-

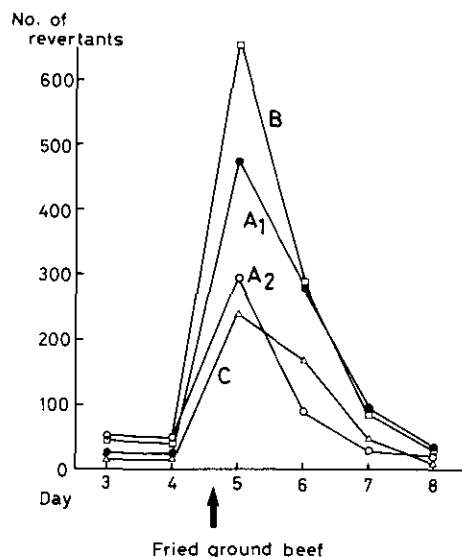


FIGURE 2. Effect of eating ground beef on fecal mutagenicity. Feces sample, which was excreted once a day, was processed as described in text, and the mutagenic activity found in fractions 2 and 3 of the CM-cellulose chromatography is plotted. General health of the donors during the experimental periods were good, and all the feces were normal in appearance. See text for the description of the donors A-C and the eating schedules.

tively, and Fractions 2-3 previously obtained from extracts of cooked ground beef had been shown to contain a mutagen identical with MeIQx in high-pressure liquid chromatography (HPLC) (3).

Small amounts of oleic acid were present in the preparation obtained after the blue-cotton adsorption (10-20  $\mu$ g oleic acid from 100-200 g feces, quantified as described) (16). These amounts were inhibitory to the mutagenicity assay (16,17), but the oleic acid could be separated from fractions containing mutagens by the CM-cellulose column chromatography (Fig. 1a).

Figure 2, curve A<sub>1</sub>, illustrates the time course of the change in the mutagenicity of fecal Fractions 2-3. The level of mutagenicity was almost zero on the third and fourth days, became high on days 5 and 6, and then resumed the original low level on day 8. The results were reproducible for the same person in another time-course study (curve A<sub>2</sub>). In experiments carried out at other times for two other donors, similar results were obtained (curves B and C). Donor B was a male Japanese, age 49, and C a male American Caucasian, age 55. Fraction 8 that can contain IQ also increased after the intake of cooked beef, but the net increase in the revertant-colony numbers was not so large as those for Fractions 2-3 (data not shown).

To investigate the nature of the mutagens arising by ingestion of the heated beef, multiple samples of feces were taken from donors A and B after they had eaten cooked ground beef, and from each sample the Fractions 2-3 was prepared. Pooled preparation of Fractions 2-3 was examined for its mutagenic characteristics by use of tester strains *S. typhimurium* TA 98 and TA 100 in the presence and absence of S-9. Fractions 2-3 showed positive mutagenicity only with TA 98 in the presence

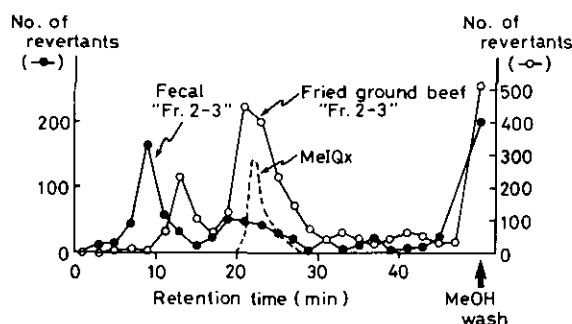


FIGURE 3. HPLC chromatograms of ground beef mutagens and fecal mutagens resulting from ingestion of the ground beef. A sample of fractions 2 and 3 of CM-cellulose chromatography, shown in Fig. 1a, was subjected to HPLC. The amount was equivalent to 33 g of the raw beef (○). Materials corresponding to fractions 2 and 3 in Fig. 1b were collected from multiple fecal samples that were excreted after ingestion of fried ground beef, and were chromatographed on HPLC (●). Column, Nucleosil C<sub>18</sub>; eluting solution, 10 mM ammonium formate at pH 6.45 in 33% methanol, and then 100% methanol for washing; flow rate, 1 mL/min. The dashed-line profile is for standard MeIQx.

of S-9: no mutagenicity was observed for TA 98 without S-9 or for TA 100 with or without S-9. Several Fractions 2-3 preparations were combined and subjected to HPLC on a reversed phase column. As Figure 3 shows, the mutagenic activity did not coincide with the peak for standard MeIQx; there were at least two mutagenic components, one that was eluted earlier than MeIQx (presumably, substances more polar than MeIQx) and another that was eluted in the 100% methanol washing (substances more hydrophobic than MeIQx). The Fractions 2-3 of the cooked ground beef gave a major peak corresponding to MeIQx (about 50% of the total activity), a minor peak that was eluted earlier than MeIQx but was different from the peak found in fecal samples (about 15% of total activity), and a substantial mutagenicity in the methanol-wash of the column (about 20% of total).

The efficiency of the extraction procedure was examined using the mutagens from cooked ground beef. The unfractionated mutagenic components of ground beef, prepared by the blue cotton adsorption, were mixed with a feces-water homogenate, and the material was processed through the procedure for extraction of mutagens from feces. When water, instead of fecal homogenate, was used for the medium, the mutagenicity recovered in two experiments was 82.8% and 89.1% for Fractions 2-3. When fecal homogenate was the medium, the recovery was much lower; 7.3% and 7.3% for Fractions 2-3. Obviously, the fecal solid materials can adsorb the mutagenic compounds efficiently. Therefore, the real content of mutagens in the feces was probably much higher than the amount detected.

Baker et al. (18) reported an increase in human urinary mutagenicity by ingestion of fried bacon. Our experiments show that the mutagens originating from cooking of ground beef can also increase the level of fecal mutagenicity.

Carcinogenicity tests for food-pyrollysate mutagens

have been carried out, and seven mutagens, including IQ, that have so far been tested were all shown to be carcinogenic to animals (19–22). The test for MeIQx is underway (H. Ohgaki, personal communication). The increase in fecal mutagenicity by ingestion of cooked meat that contains MeIQx, and probably IQ, raises the possibility that it is related to the incidence of colon cancer.

## Concluding Remarks

The use of blue cotton has made the treatment of human feces into a simple and nonlaborious one. It was previously shown that urinary mutagenicity can be easily monitored by the blue-cotton adsorption (23). Blue cotton can also be used in monitoring mutagens in various foods (24). We have prepared cellulose powder and silica gel to which sulfonated copper-phthalocyanine moieties are covalently attached. Since individual mutagens have their own affinity to these materials, it is possible to achieve separation among mutagens bearing similar structures by column chromatography through these materials (unpublished work).

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